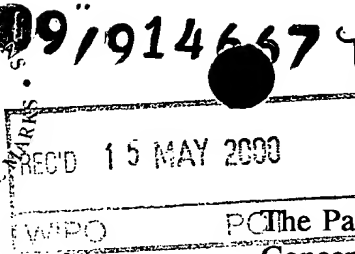




GB00/1198



INVESTOR IN PEOPLE

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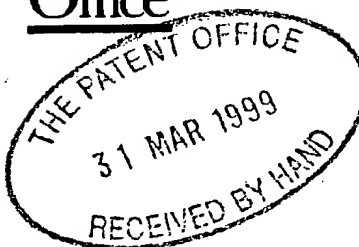
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Dated 28/4/00

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# Request for the grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)



The Patent Office

Cardiff Road  
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Gwent NP9 1RH

1. Your reference

REP06088GB

2. Patent application number

(The Patent Office will fill in this part)

9907415.5

31 MAR 1999

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Chirotech Technology Limited  
Cambridge Science Park  
Milton Road  
Cambridge  
CB4 4WE  
United Kingdom

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

GB

7410996001

4. Title of the invention

BIOCATALYST AND ITS USE

5. Name of your agent (if you have one)

GILL JENNINGS & EVERY

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Broadgate House  
7 Eldon Street  
London  
EC2M 7LH

Patents ADP number (if you know it)

745002

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

Priority application number  
(if you know it)

Date of filing  
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing  
(day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

YES

- a) any applicant named in part 3 is not an inventor
  - b) there is an inventor who is not named as an applicant, or
  - c) any named applicant is a corporate body.
- See note (d))

# Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form.  
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Continuation sheets of this form

Description	8
Claim(s)	2
Abstract	0
Drawing(s)	0

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (Patents Form 7/77)

Request for preliminary examination and search (Patents Form 9/77)

Request for substantive examination (Patents Form 10/77)

Any other documents (please specify)

11. For the Applicant  
Gill Jennings & Every

I/We request the grant of a patent on the basis of this application.

Signature

Date

31 March 1999

12. Name and daytime telephone number of person to contact in the United Kingdom

PERRY, Robert Edward  
0171 377 1377

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## BIOCATALYST AND ITS USE

### Field of the Invention

This invention relates to a process for the production of optically active  $\beta$ -lactams  
5 by way of resolution of the racemic  $\beta$ -lactam using a microbial whole-cell preparation.

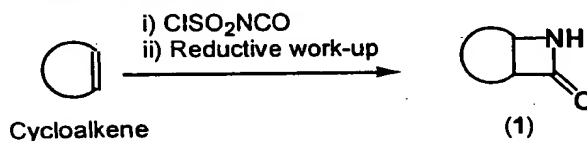
### Background of the Invention

The utility of the  $\beta$ -lactam ring as a structural feature in biologically important  
compounds is well established, as is the synthetic utility of  $\beta$ -lactams (for lead references  
see Georg. G, *The Organic Chemistry of  $\beta$ -Lactams.*, VCH, 1993). For example, the  
10 utility of  $\beta$ -lactams as masked  $\beta$ -amino acids is illustrated by their use as precursors to the  
C-13 taxol side chain (Brieva *et al.*, *J. Org. Chem.*, 1993, 58, 1068). The utility of alicyclic  
 $\beta$ -amino acids is described below. Conversely,  $\beta$ -amino acids can cyclised to the  
corresponding  $\beta$ -lactams, typically by use of carbodiimide reagents, as demonstrated in  
pioneering work by Sheehan in the penicillin field (Sheehan *et al.*, *J. Am. Chem. Soc.*,  
15 1962, 84, 2983).

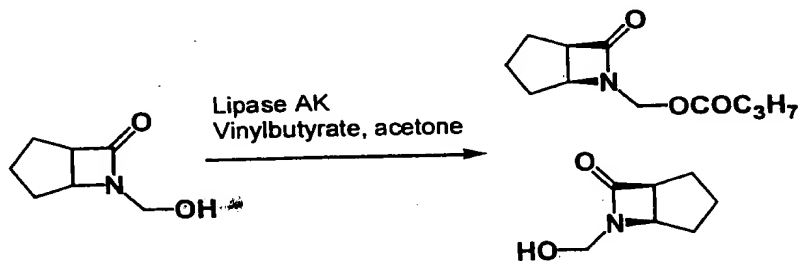
For such applications end products are often chiral molecules and it is preferable  
that these are accessible in the form of single enantiomers. This feature is also utilised  
in the design of stereodefined scaffolds, to generate single enantiomer compounds  
libraries for initial lead identification as part of a drug discovery programme.

20 Alicyclic  $\beta$ -amino acids are important intermediates or end-points with  
biological or pharmaceutical activity. An example can be found in cispentacin (FR  
109615), (-)-(1*R*,2*S*)-2-aminocyclopentanecarboxylic acid, which is a natural product  
with potent antifungal properties (Konishi *et al*, *Journal of Antibiotics*, 1989, 42, 1576;  
Iwamoto *et al*, *Journal of Antibiotics*, 1990, 43, 1; Kawabata *et al*, *Journal of*  
25 *Antibiotics*, 1990, 43, 513). The compound is synthesised as a natural product in  
*Streptomyces setonii* and *Bacillus cereus*, and is inhibitory to strains of the *Candida*.  
The same amino acid has also been used to probe the relationship between structure and  
taste in L-aspartyl dipeptides, where the absolute structure of the  $\beta$ -amino acid strongly  
affected the taste of the dipeptide.

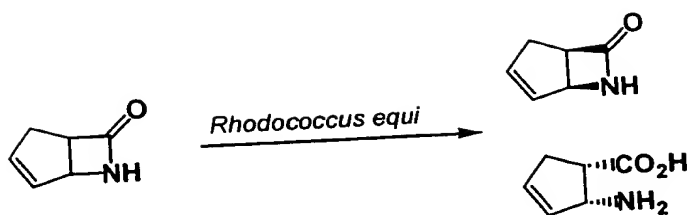
Routes for the synthesis of optically pure alicyclic  $\beta$ -amino acids have been described, for example by the enzyme catalysed solvent-based bioresolution of the amino carboxyl esters (Kanerva *et al.*, *Tetrahedron: Asymmetry*, 1996, 7, 1705). Such racemic amino esters are readily synthesised from the  $\beta$ -lactam of formula (1), which are themselves synthesised by cycloaddition of the appropriate cyclic alkene and chlorosulfonylisocyanate, followed by reductive work-up using sodium sulfite:



It is generally preferable to resolve enantiomers as early as possible in a synthetic sequence, to avoid having to perform the chemistry on the racemate at twice the scale as for the enantiomer. Thus, it is useful to be able to access  $\beta$ -lactam precursors of  $\beta$ -amino acids in resolved form. One approach is to derivatise the  $\beta$ -lactam to the *N*-hydroxymethyl- $\beta$ -lactam using paraformaldehyde (Csomós *et al.*, *Tetrahedron: Asymmetry*, 1996, 7, 1789). The resulting primary alcohols can then be resolved using a lipase, for example to access the *O*-acylated precursor to cispentacin, as depicted below. Besides the extra derivatisation and deprotection required, the resolution with lipase AK requires high enzyme loading and chromatographic separation of the products making this general approach unattractive at scale.



A more direct approach would be to resolve the lactam itself, thereby removing the separation problem, since the resulting amino acid is soluble in buffer, whilst the substrate can be extracted into solvent. This has been shown in one instance (Evans *et al.*, *J. Chem. Soc., Perkin Trans. 1*, 1991, 2276) where *Rhodococcus equi* was used to provide (1*R*, 5*S*)-6-azabicyclo[3.2.0]hept-3-ene-7-one:



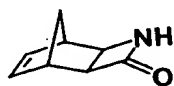
However, this biotransformation is an exceedingly slow process and is therefore not viable for operation on a commercial scale; 980 mg of whole-cell paste was needed to resolve 340mg of racemate in 212 hrs, in order to recover unreacted lactam of >99% ee. Thus there is a requirement for more effective  $\beta$ -lactamase biocatalysts, especially those which extend the range of fused  $\beta$ -lactams available as single enantiomers.

#### Summary of the Invention

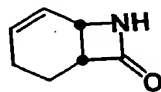
The present invention is based on the discovery of a novel lactamase biocatalyst of general utility in efficient to access a range of synthetically useful  $\beta$ -lactams and the corresponding  $\beta$ -amino acids. A preferred embodiment of the invention is the application of this methodology to the preparation of novel single enantiomer cyclohexene-fused  $\beta$ -lactams.

#### Description of the Invention

For identification of a suitable biocatalyst, a microbial screen was targeted at hydrolysis of racemic lactams 2 and 3. Hitherto, neither compound has been prepared in enantiomerically enriched form. Lactams 2 and 3 are conveniently synthesised by cycloaddition of chlorosulfonyl isocyanate with norbornadiene and cyclohexa-1,3-diene respectively (Stájer *et al.*, *Tetrahedron*, 1984, 40, 2385; Malpass *et al.*, *J. Chem. Soc., Perkin Trans. 1*, 1991, 2276).



(2)

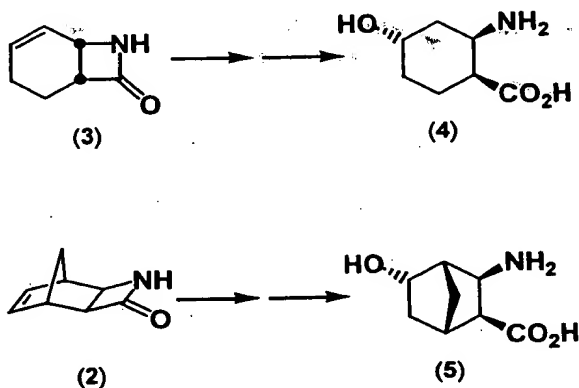


(3)

A total of 400 microbial strains were screened for hydrolysis of the above lactams. To our surprise, one strain of *Pseudomonas putida* not only hydrolysed the lactam, but did so selectively, with much higher activity than observed for the prior art strain of *Rhodococcus* (Evans *et al.*, reference as above). Thus for lactam (2), when

stirred with an equal weight of cell paste in phosphate buffer at 25°C, resolution was complete after 15 hours (>98% ee residual substrate), and the enantiomerically pure lactam extracted into ethyl acetate in 38% yield. Similarly for lactam (3), after incubation with an equal weight of cells for 21 hours in buffer, the ee of residual lactam was >90%. Recovery of the enantiomerically enriched  $\beta$ -amino acids from aqueous solution is facilitated by conversion to *N*-Boc derivatives under standard conditions, followed by solvent extraction.

Enantiomerically enriched products obtained from the bioresolution of lactams 2 and 3 can be used to prepare information-rich chiral scaffolds for elaboration as into single enantiomer compounds libraries. The alkene functionality is amenable to a range of transformations, especially through reaction with oxidants. For example, conversion of 3 to the chiral scaffold 4 gives three points for structural elaboration into defined regions of 3-dimensional space. Similarly, the more rigid scaffold 5 can be prepared via bioresolution of 2.



15

In summary, the present invention embodies a novel  $\beta$ -lactamase biocatalyst from *Pseudomonas putida* combining the key attributes of high enantioselectivity with superior catalytic activity to  $\beta$ -lactamases described previously. Use of this biocatalyst allows certain single enantiomer  $\beta$ -lactam to be prepared for the first time in synthetically useful amounts.

20

The following examples illustrate the invention.



**Example 1****Preparation of 96 well culture plate SCL0003.**

5 Glycerol stocks of 96 bacterial strains (obtained from the applicant's strain collection) were used to inoculate 1.0 ml Tryptone soya broth (Oxoid CM129) per well in 2.2ml 96 well plates (Advanced Biotechnologies AB-0661). These were then shaken at 25°C on a Heidolph Titramax 1000 incubator at max rpm for 45 hours. The cells were harvested by centrifugation at 1000g, 4°C, for 10 minutes and the cell pastes were stored at -20°C for future screening.

**10 Example 2****Screening of the 96 well culture plate, SCL0003, against norbornadiene and cyclohexa-1-3-diene lactam derivatives.**

Cell pastes of the 96 well culture plates SCL0003 were resuspended in 0.5 mls of 20g.l<sup>-1</sup> of substrate in 0.1M KH<sub>2</sub>PO<sub>4</sub>, pH 7.0 (13.6g KH<sub>2</sub>PO<sub>4</sub> in 1.0l water, adjusted to pH 7.0 with 12M NaOH) and then shaken at 25°C on a Heidolph Titramax 1000 incubator at max rpm for 40 (on the norbornadiene lactam derivative) to 66 hours (on the cyclohexa-1-3-diene lactam derivative). Reactions were stopped by diluting 1 in 10 in a 1:1 mix of MeOH:10mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.0 (1.36g KH<sub>2</sub>PO<sub>4</sub> in 1.0l water, adjusted to pH 7.0 with 12M NaOH). These were then assayed by HPLC. A 15cm 5µ Hichrom KR100 C8 Column was used with a running buffer of a 1:1 mix of MeOH:10mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.0 (1.36g KH<sub>2</sub>PO<sub>4</sub> in 1.0l water, adjusted to pH 7.0 with 12M NaOH) at a flow rate of 1.0 ml.min<sup>-1</sup>. Detection was at 210 nm. Results showed that CMC 103381, *Pseudomonas putida* had reached 45% conversion on the norbornadiene lactam derivative and 43% conversion on the cyclohexa-1-3-diene lactam derivative. CMC 20 103381 was deposited at NCIMB Ltd on 31.03.99. The provisional accession number is 41013. 25

**Example 3****Preparation of CMC 103381 cell pastes.**

30 A 10µl loopful of colony of CMC 103381 (from agar plate) was used to inoculate seed flasks (100ml Tryptone soya broth (Oxoid CM129) per 500ml Erlenmeyer flask). These were shaken at 25°C, 300rpm in a temperature controlled

shaker (New Brunswick G-25 with 1 inch throw) for 23 hours. A 0.67% v/v inoculum was used to inoculate the fermenters. The fermenters used were 3.0l Applikon fermenters with 1.5l of the following media per fermenter: 15 g.l<sup>-1</sup> Yeast extract (Oxoid L21), 8 g.l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 7g.l<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 1g.l<sup>-1</sup> MgSO<sub>4</sub>.7H<sub>2</sub>O, 1g.l<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 ml.l<sup>-1</sup> Trace elements solution, 1.0 ml.l<sup>-1</sup> polypropylene glycol (Merck 29767 6Y), 20 g.l<sup>-1</sup> glucose. The trace elements solution was 250 ml.l<sup>-1</sup> 85% 36% HCl, 3.6 g.l<sup>-1</sup> CaCl<sub>2</sub>.2H<sub>2</sub>O, 2.0 g.l<sup>-1</sup> ZnO, 0.85 g.l<sup>-1</sup> CuCl<sub>2</sub>.2H<sub>2</sub>O, 2.0 g.l<sup>-1</sup> MnCl<sub>2</sub>.4H<sub>2</sub>O, 5.4 g.l<sup>-1</sup> FeCl<sub>3</sub>.6H<sub>2</sub>O, 2.4 g.l<sup>-1</sup> CoCl<sub>2</sub>.2H<sub>2</sub>O, 4.8 g.l<sup>-1</sup> Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O, 0.3 g.l<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>. Control was with Biolab II controllers (Brighton systems). The fermenters were grown at 25°C, pH control between 6.9 and 7.1 (with 5M NaOH/H<sub>3</sub>PO<sub>4</sub>) 800 rpm, air flow at 1.0 ml.min<sup>-1</sup> for 40 hours. Cell pastes were harvested by centrifugation at 9,000 g, 4°C for 20 minutes and were stored at -20°C for future use.

#### Example 4

##### 15 Synthesis of *exo*-3-Aza-tricyclo[4.2.1.0<sup>2,5</sup>]non-7-en-4-one

A solution of 20.3g of norbornadiene in 100ml of dichloromethane was slowly added, with stirring, to a solution of 19.1ml of chlorosulfonyl isocyanate in 40ml of dichloromethane. Upon addition, the solution turned red and gradually darkened to a deep purple colour. After the addition was complete, the mixture was left stirring for a further 30 minutes. The solution was then slowly added to a stirred mixture of 75ml of 33% sodium sulfite solution and 50ml dichloromethane, keeping the temperature at 25°C. The reaction mixture was left stirring for a further 30 minutes after the addition was complete. The organic layer was then separated from the aqueous and dried over magnesium sulfate. Removal of solvent by rotary evaporator yielded 18g, 61%, of a white solid. GC-MS : indicated m/z: 135 (M<sup>+</sup>), 107, 91, 70.

#### Example 5

##### Bioresolution of *exo*-3-Azatricyclo[4.2.1.0<sup>2,5</sup>]non-7-en-4-one using *P. putida* CMC 103381

30 Into a 125ml conical flask was placed 2g lactam, 40ml of 50mM phosphate buffer, pH 7 and 0.5g of *P. putida* CMC103381. The reaction vessel was placed inside

a heated jacket to ensure that the temperature remained at 25°C. The reaction mixture was gently stirred and after 24 hours a further 1.5g of *P.putida* cells were added to the reaction mixture. After a total reaction time of 39 hours an aliquot from the reaction was found by chiral GC to contain only a single enantiomer of the cyclic  $\beta$ -lactam. The reaction was halted after 46 hours, the enzyme cells were spun out by centrifugation (3400 rpm) and the resulting pellet was washed with distilled water and respun. The supernatant was extracted with 2 x 70ml ethyl acetate, the organic extracts were combined and dried over magnesium sulfate. Removal of solvent by rotary evaporator yielded 770mg of a white solid,  $[\alpha]_D = -91^\circ$  (20°C, MeOH). Chiral GC analysis was carried out using the Chirasil Dex-CB column: Retention time: 4.77 minutes, enantiomeric excess >95%.

#### Example 6

##### Synthesis of 7-azabicyclo[4.2.0]-oct-4-en-8-one.

A solution of 10ml of cyclohexa-1,3-diene in 35ml of dichloromethane was slowly added, with stirring, to a solution of 7.4ml of chlorosulfonyl isocyanate in 150ml of dichloromethane. Upon addition, the solution turned red and gradually darkened to a deep purple colour. After the addition was complete, the mixture was left stirring for a further 5 minutes. The solution was then slowly added to a stirred mixture of 100ml of 25% sodium sulfite solution and 50ml dichloromethane. The reaction mixture was left stirring for a further 20 minutes after the addition was complete. The organic layer was then separated from the aqueous and dried over magnesium sulfate. Removal of solvent by rotary evaporator yielded 3.4g, 33%, of a yellow oil which solidified on standing. GC-MS : indicated m/z: 123 ( $M^+$ ), 94, 80

$^1H$  NMR ( $CDCl_3$ ): 6.3-5.8 (2H; m), 4.1 (1H; t), 3.5 (1H; brs), 2.3-1.3 (4H; m).

#### Example 7

##### Bioresolution of 7-azabicyclo[4.2.0]-oct-4-en-8-one.

Into a 100ml conical flask was placed 1.49g lactam, 40ml of 50mM phosphate buffer, pH 7 and 1.41g of *P.putida* CMC103381. The reaction vessel was placed within a heated jacket and the temperature was maintained at 30°C. The reaction mixture was stirred continuously for 22 hours, after which time an aliquot was taken and analysed by

chiral GC to determine whether the resolution was complete. The aliquot was extracted with ethyl acetate and derivatised using TFAA. The resulting chiral GC chromatogram indicated lactam with an e.e. of 93%. The reaction was halted, enzyme cells were spun off by centrifuge (3400 rpm) and the supernatant was extracted with 3 x 50ml of ethyl acetate.

- 5 The organic phase was dried over magnesium sulfate and solvent was removed by rotary evaporator to yield 460mg of an off-white solid,  $[\alpha]_D = -105.5^\circ$  (20°C; MeOH). Chiral GC analysis was carried out using a Chirasil Dex-CB column: Retention times: 15.46 mins (major enantiomer), 15.79 mins (minor enantiomer) Enantiomeric excess: 93%.

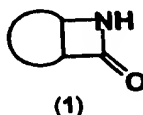
- 10 The amino acid produced from the bioresolution of 7-azabicyclo[4.2.0]-oct-4-en-8-one was isolated in two steps as the hydrochloride salt.

- The 15ml of buffered solution remaining from the bioresolution described above contain 1g (7.1mmol) of amino acid (maximum). To the stirred buffer solution was slowly added, at 10°C, 1.55g (7.1mmol) of dibutyl dicarbonate dissolved in 20ml of THF. The pH of the solution was maintained at 9 by addition of 3M NaOH solution.
- 15 The reaction mixture was allowed to warm up to room temperature and left stirring overnight. The reaction was halted after 18 hours and THF was removed by rotary evaporator. The aqueous reaction mixture was acidified to pH3 with 10% potassium hydrogen sulfate solution. The mixture was then extracted with 3 x 50ml of ethyl acetate and the combined organic extracts were dried over magnesium sulfate. Removal
- 20 of solvent by rotary evaporator yielded 1.1g, 62% of a white solid.

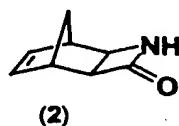
- Into a 25ml conical flask was placed 0.71g (2.81mmol) of Boc protected amino acid, dissolved in 15ml THF. The stirred reaction mixture was acidified to pH 3 with 3M HCl solution and the reaction was vigorously stirred for 3 hours at ambient temperature. The reaction mixture was left to stand overnight and extracted with 10ml
- 25 ethyl acetate. The aqueous phase was concentrated under vacuum to yield 480mg, 87% yield of an off white solid.  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ): 6.05-5.85 (1H; m), 5.65-5.50 (1H; m), 3.9 (1H; brs), 2.85 (1H; dt), 2.15-1.65 (4H; m).

CLAIMS

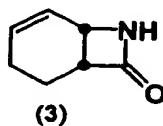
1. A process for the preparation of an enantiomerically enriched  $\beta$ -lactam which comprises enantioselective hydrolysis of the corresponding racemic  $\beta$ -lactam in the presence of lactamase enzyme from a microorganism having the characteristics of that available as the *Pseudomonas putida* strain, NCIMB 41013.
2. A process according to claim 1 wherein the lactamase enzyme is used in a form selected from one of the following: an isolated and purified enzyme, a cell paste, or intact cells.
3. A process according to either of claims 1 & 2 wherein the lactam is a fused polycyclic compound of the type represented by formula (1), wherein ring A is any monocyclic or any polycyclic ring, optionally substituted with one or more non-interfering groups.



4. A process according to claim 3 which additionally comprises isolation of an enantiomerically enriched  $\beta$ -amino acid produced by hydrolysis of the  $\beta$ -lactam substrate.
5. A process according to claim 4 wherein the isolated  $\beta$ -amino acid is then subjected to a condensation reaction to reform the  $\beta$ -lactam ring.
6. A process according to either of claims 3 and 4 wherein the  $\beta$ -lactam is 3-azatricyclo[4.2.1.0<sup>2,5</sup>]non-7-en-4-one (2).



7. A process according to either of claims 3 and 4 wherein the  $\beta$ -lactam is 7-azabicyclo[4.2.0]oct-4-en-8-one (3).



8. An enantiomer of 3-azatricyclo[4.2.1.0<sup>2,5</sup>]non-7-en-4-one (2), at least substantially free of the opposite enantiomer, wherein the enantiomeric excess is not less than 80%, preferably not less than 95%.
9. 3-Azatricyclo[4.2.1.0<sup>2,5</sup>]non-7-en-4-one (2) according to claim 7, as the  
5 levorotatory enantiomer.
10. An enantiomer of is 7-azabicyclo[4.2.0]oct-4-en-8-one (3), at least substantially free of the opposite enantiomer, wherein the enantiomeric excess is not less than 80%, preferably not less than 95%.
11. 7-Azabicyclo[4.2.0]oct-4-en-8-one (3) according to claim 7, as the levorotatory  
10 enantiomer.
12. A lactamase enzyme from a microorganism having the characteristics of that available as the *Pseudomonas putida* strain, NCIMB 41013.